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Cyclosporin A suppresses the expression of the interleukin 2 gene by inhibiting the binding of lymphocyte-specific factors to the IL-2 enhancer

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Cyclosporin A (CsA), a powerful immunosuppressive drug, inhibits the synthesis of lymphokines in T lymphocytes at the level of gene transcription. Using protein extracts from El4 lymphoma cells we show that the binding of lymphocyte-specific factors interacting with the two so-called purine boxes (Pu-boxes) of the interleukin 2 (IL-2) enhancer are missing in CsA-treated cells. The CsA-sensitive factors are newly synthesized upon induction. The most prominent factor consists of 45 kd polypeptides and contacts both Pu-boxes at the two central G residues within the identical core sequence AAGAGGAAAA. The CsA-mediated suppression of factor binding to the Pu-boxes correlates well with functional studies in which the inducible, T cell-restricted proto-enhancer activity of Pu-boxes was selectively repressed by CsA. These observations support the conclusion that the suppression of factor binding to the Pu-boxes by CsA impairs the activity of IL-2 and of further lymphokine genes, thereby inhibiting the synthesis of lymphokines in T lymphocytes.

Key words: cyclosporin A/interleukin 2/T lymphocytes/transcription factors

Introduction

Cyclosporin A (CsA), a cyclic unadecapeptide of fungal origin, is a powerful immunosuppressive drug that is widely used in transplantation medicine. It is generally accepted that the immunosuppressive effect of CsA is due to the inhibition of lymphokine synthesis in T lymphocytes (Drugge and Handschuhmacher, 1988; Kahan, 1989). The repression of lymphokine synthesis by CsA occurs at the level of gene transcription (Elliott *et al.*, 1984; Krönke *et al.*, 1984), but there are, to date, no experimental data on a specific nuclear target for CsA in T lymphocytes.

To study the effect of CsA at the molecular level, we chose the interleukin 2 (IL-2) gene. The activity of the IL-2 gene plays a pivotal role in the activation and differentiation of T lymphocytes and is suppressed by low doses of CsA (0.1 μg/ml) (see Smith, 1988; Crabtree, 1989). *In vivo*, the IL-2 gene is activated as a result of the interaction of presented antigens with the T cell receptor complex, and the synthesized IL-2 triggers, in turn, T cell growth and the secretion of further lymphokines. This effect is mediated by the interaction of IL-2 with a high-affinity receptor consisting of (at least) two polypeptide chains (Smith, 1988). High-affinity IL-2 receptors are also expressed on pro-thymocytes, and it seems very likely that IL-2 plays an important role in early T lymphocyte development within the thymus as well (Toribio *et al.*, 1989).

In T lymphoma cells the activity of the IL-2 gene is controlled by a 275 bp-long enhancer located within its immediate upstream region (Fujita *et al.*, 1986; Durand *et al.*, 1987; Serfling *et al.*, 1989). Transfection experiments have shown that this enhancer spans the majority of DNA sequence elements necessary for the T lymphocyte-specific induction of the IL-2 gene by mitogens. This conclusion is supported by investigations of transgenic mice in which chimeric test genes carrying the IL-2 enhancer are exclusively expressed in activated T lymphocytes (Crabtree, 1989).

In both the murine and human systems the IL-2 enhancer is bound by a variety of ubiquitous and lymphocyte-specific factors (Brunvand *et al.*, 1988; Durand *et al.*, 1988; Shaw *et al.*, 1988; Shibuya *et al.*, 1988; Hoyos *et al.*, 1989; Serfling *et al.*, 1989; Shibuya and Taniguchi, 1989). As described for the enhancers of many other cell type-specific genes (see Renkawitz, 1989), the cooperative activity of ubiquitous and cell-specific factors seem to control the activity of the IL-2 gene enhancer as well.

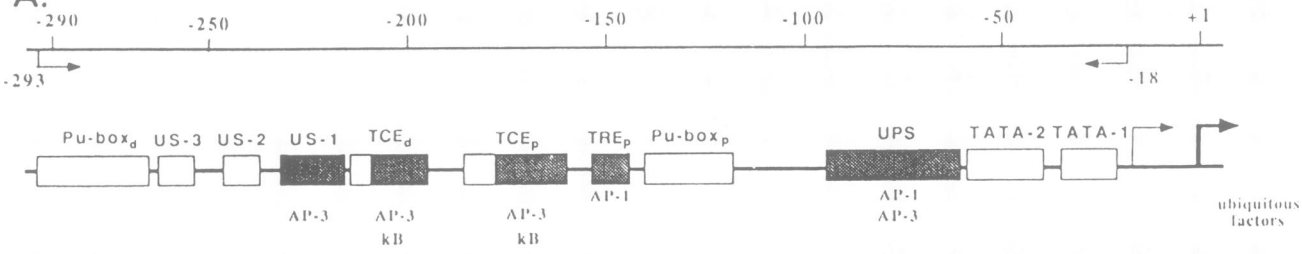
CsA selectively inhibits the activity of many lymphokine genes in activated T lymphocytes, such as the IL-2, IL-4 and γ-interferon genes (see Zipfel *et al.*, 1989). These observations suggest that CsA inhibits the generation and/or the activity of inducible, lymphocyte-specific *trans*-acting factors interacting with regulatory DNA sequences of CsA-sensitive genes. We will show here that indeed, this is the case. CsA prevents the binding of T lymphocyte-specific factors to the two Pu-boxes of the IL-2 enhancer. In El4 lymphoma cells, these factors are newly synthesized upon induction and probably also interact with further lymphokine genes such as the IL-4 gene. Our data support the view that CsA inhibits the synthesis of these Pu-box factors which seem to play a crucial role in the regulation of lymphokine gene transcription.

Results

The identification of CsA-responsive elements within the IL-2 enhancer

When chimeric plasmids containing the IL-2 enhancer (Figure 1A) upstream of the thymidine kinase (tk) promoter/chloramphenicol acetyltransferase (CAT) reporter gene are transfected into El4 murine lymphoma cells, the synthesis of CAT is inducible with the phorbol ester 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) and the plant lectin concanavalin A (ConA). Addition of CsA (0.1 – 1 μg/ml) to the TPA/ConA-containing cell culture medium suppresses CAT synthesis (Figure 1B). Under the same conditions the expression of a $5\times TRE_{Coll}$-CAT plasmid containing five AP-1 binding sites from the promoter of the human

A.



B.

Construct	Il2-Sequence	Induction Ratio (% of Acetylation) -CsA	+CsA	No. Experiments
pIlCAT2/1+	-7/-293	106 (50)	6 (3)	4
5xTRE$_{Coll}$	--	16 (3.5)	32 (4)	3
5xTCE$_d$ + tk promoter	-191/-215 (5x)	103 (25)	70.5 (13.5)	2
5xTCE$_d$ +Il2-Prom.	-191/-215 (5x) +48/-104	33.5 (11)	51 (13)	2
pIlCAT2/1-L (3x)	-180/-293 (3x)	407 (91)	10 (5)	2
pIlCAT2/1-M (3x)	-200/-293 (3x)	56 (48)	3 (2)	3
pIlCAT2/1+B	-7/-250	25 (18)	2 (1)	3
pIlCAT2/1+D	-7/-200	6 (7)	1.8 (1)	3

C.

4xTCE$_d$ + tk promoter	-191/-215 (4x)	16 (8)	14 (6)	2
4xPu-box$_d$	-264/-292 (4x)	22 (6)	2 (0.3)	3

Fig. 1. Localization of CsA-responsive DNA elements within the IL-2 enhancer. **A**. Organization of the T lymphocyte-specific inducible enhancer of the murine IL-2 gene. The binding sites for *trans*-acting factors are indicated by boxes. Filled boxes represent the binding sites of the ubiquitous factors AP-1, AP-3 and NF-$\varkappa$B, and open boxes the binding sites for lymphoid-specific factors. Purified AP-1 also binds to the TREd (around position −185) and upstream sites around −225 to −270. Since these sites are not protected by crude extracts from E14 and HeLa cells, they are not indicated. Likewise, the binding of octamer-like factors to the UPS and further enhancer sites (see Emmel *et al.*, 1989; Shibuya and Taniguchi, 1989) is not indicated. For the detection of factor binding, purified AP-1 and AP-3 from HeLa cells (Angel *et al.*, 1987; Mercurio and Karin, 1989) and enriched AP-1 and AP-3 from E14 cells, for the binding of NF-$\varkappa$B, purified NF-$\varkappa$B from human placenta (Baeuerle and Baltimore, 1989) was used. Abbreviations: TATA-1,2: TATA boxes 1 and 2; UPS: upstream promoter site; Pu-box$_{p,d}$: proximal and distal purine box; TRE$_p$: proximal TPA responsive element; TCE$_{p,d}$: proximal and distal T cell element; US-1, -2 and -3: upstream sites 1, 2 and 3. **B**. The effect of CsA on the activity of different IL-2 enhancer constructs after transfection into E14 cells. The construction of pIlCAT plasmids and of 5×TCEd has been described (Serfling *et al.*, 1989; see Materials and methods). 5×TRE$_{Coll}$ contains five copies of the AP-1 binding site from the human collagenase promoter (Angel *et al.*, 1987) in pBLCAT2. The 'Induction Ratio' was calculated by dividing the percentage of chloramphenicol acetylation of induced cells (or of induced and CsA-treated cells) by the percentage of acetylation of uninduced cells. The '% of acetylation' corresponds to the acetylated chloramphenicol determined for the stimulated cells. **C**. The effect of CsA on the activity of constructs 4×Pu-box$_d$ and 4×TCEd containing four copies of distal Pu-box (from position −264 to −292) or of distal TCEd (−191 to −215). In these experiments, 0.1 μg CsA was added 3 h after the addition of TPA/ConA.

collagenase gene (Angel *et al.*, 1987) is not inhibited. Surprisingly, the expression of 5×TCEd constructs was also not (or only weakly) affected by CsA (Figure 1B). 5×TCEd harbours five copies of the IL-2 enhancer sequence from positions −191 to −215, and its activity is restricted to T cells (Serfling *et al.*, 1989). The resistance of TCEd against CsA shows that not all IL-2 enhancer motifs are a target of CsA-responsive factors.

To identify the CsA-responsive elements within the IL-2 enhancer, we tested several enhancer mutants. Trimer constructs of the distal enhancer segment (from −180 or −200 to −293) showed a CsA sensitivity similar to that of the entire enhancer (Figure 1B). Likewise, 5′ deletion mutants which lack the sequences upstream of positions −250 or −200 were found to be inhibited by CsA, while 5×TCEd constructs containing the IL-2 promoter instead of the tk promoter were CsA resistant (Figure 1B). These data suggest the existence of at least two CsA-responsive elements, one element located within the distal enhancer fragment (between −210/−215 and −293), and one within the central enhancer fragment (between −104 and −190).

The distal and central enhancer fragments share two repeated sequence motifs, the two TCEs (TCEp and TCEd) and purine boxes (Pu-b$_p$ and Pu-b$_d$) (Figure 1A). Since the TCEd appeared to be CsA resistant, we assumed that the Pu-boxes sharing the identical decamer AAGAGGAAAA are the CsA-sensitive DNA elements. To test this hypothesis, we inserted four copies of the distal Pu-box into pBLCAT2 and tested the construct 4×Pu-b$_d$ in El4 cells. As predicted, the inducible activity of 4×Pu-b$_d$ was inhibited by very low

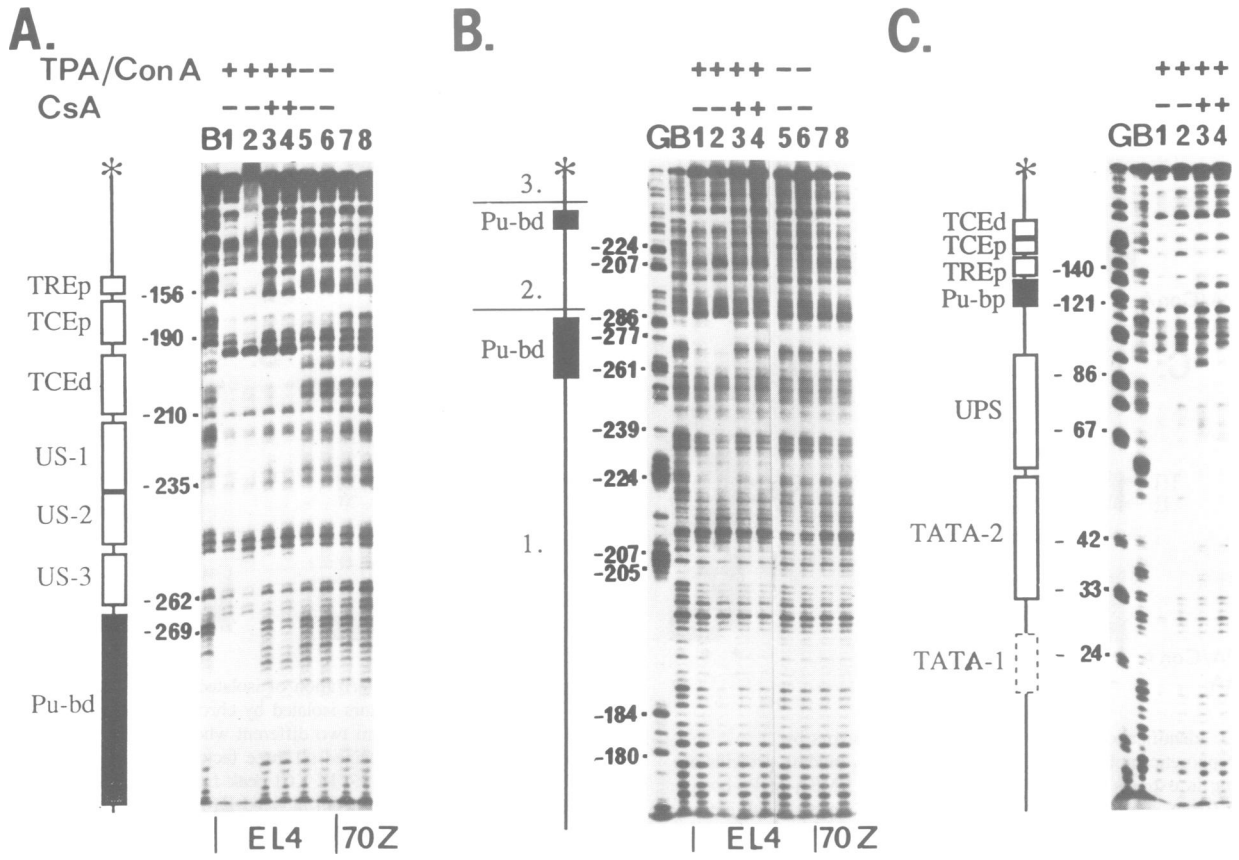


Fig. 2. Cyclosporin A inhibits the binding of factors to the Pu-boxes of the IL-2 enhancer. **A** and **B**: DNase I footprint protection of both strands of the distal IL-2 enhancer DNA. Note the protection over the Pu-b_d (filled boxes) by the extracts from induced E14 cells (lanes 1 and 2), and the lack of protection by the extract from induced and CsA-treated E14 cells (3 and 4), uninduced cells (5 and 6) and from LPS-induced 70Z pre-B cells (7 and 8). **A**. The *Hin*dIII−*Bam*HI fragment of construct pIlCAT2/1+ (see Figure 1), labelled at the *Hin*dIII site. Lanes 1 and 2: 25 μg (lane 1) and 60 μg (lane 2) protein from E14 cells induced for 4 h by 10 ng/ml TPA and 2.5 μg/ml ConA. Lanes 3 and 4: 25 μg (3) and 60 μg (4) protein from E14 cells treated for 4 h by TPA/Con A and 1 μg/ml CsA. Lanes 5 and 6: 25 μg (5) and 60 μg (6) protein from uninduced E14 cells. Lanes 7 and 8: 25 μg (7) and 60 μg (8) protein from 70Z pre-B cells induced for 20 h by 5 μg/ml LPS. B: control incubation with 25 μg bovine serum albumin. **B**. The *Hin*dIII−*Bam*HI fragment from construct pILCAT2/1-L3x (Serfling *et al.*, 1989) containing three copies of the IL-2 enhancer fragment from position −180 to −293, oriented opposite to the transcription direction of the CAT gene. The fragment was labelled at the *Hin*dIII site. In lanes 1 and 2: 25 μg or 60 μg protein extract from induced E14 cells; lanes 3 and 4: 25 or 60 μg extract from induced and CsA-treated E14 cells; lanes 5 and 6: 25 or 60 μg extract from uninduced E14 cells; lanes 7 and 8: 25 or 60 μg extract from LPS-induced 70Z pre-B cells. G: G-specific sequencing reaction. **C**. DNase I footprint protection of the proximal region of the IL-2 enhancer. Note the protection of Pu-b_p by the extract from induced E14 cells (lanes 1 and 2) and the lack of protection by extract from CsA-treated cells (lanes 3 and 4). Lanes 1 and 2: 25 μg/60 μg protein of an extract from induced E14 cells. Lanes 3 and 4: 25 μg/60 μg protein of an extract from induced and CsA-treated E14 cells.

doses of CsA (0.1 μg/ml), whereas that of the construct 4×TCEd appeared to be CsA-resistant under the same conditions (Figure 1C).

CsA impairs the binding of factors to the Pu-boxes

The two Pu-boxes are bound by lymphocyte-specific factors (Serfling *et al.*, 1989; Figure 2A and B). When extracts from induced E14 cells were analysed in DNase I footprint protection experiments, the Pu-box_d sequences between −265 and −293 were fully protected on both DNA strands (see lanes 1 and 2 in Figure 2A and B). In extracts of CsA-treated El4 cells these factors appeared to be missing (Figure 2A and B, lanes 3 and 4). The Pu-b_d sequences were also not protected when extracts were assayed from uninduced El4 cells and LPS-stimulated 70Z pre-B cells (Figure 2A and B, lanes 5−8). In the latter extracts, however, several other factors (e.g. AP-1 and the TCE factors) were also missing, while in the extracts from CsA-treated El4 cells the binding of all the other factors remains unchanged (Figure 2A, lanes 3 and 4).

CsA also suppresses the binding of factors to the proximal Pu-box. This is shown in Figure 2C (lanes 3 and 4) where no protection is detectable over the Pu-b_p DNA when extracts from CsA-treated cells were used. Subtle differences between the extracts could also be observed over the regions of TATA-box 2 and a part of the UPS motif, around position −94. Both areas contain purine stretches of six or seven nucleotides to which Pu-box factors could bind *in vitro*.

Multiple factors bind to the two Pu-boxes

Electrophoretic mobility shift assays (EMSA) were employed to characterize the Pu-box factors in more detail. After incubation of Pu-b_d oligonucleotides with extracts from induced El4 cells, the three complexes I, II and III appeared

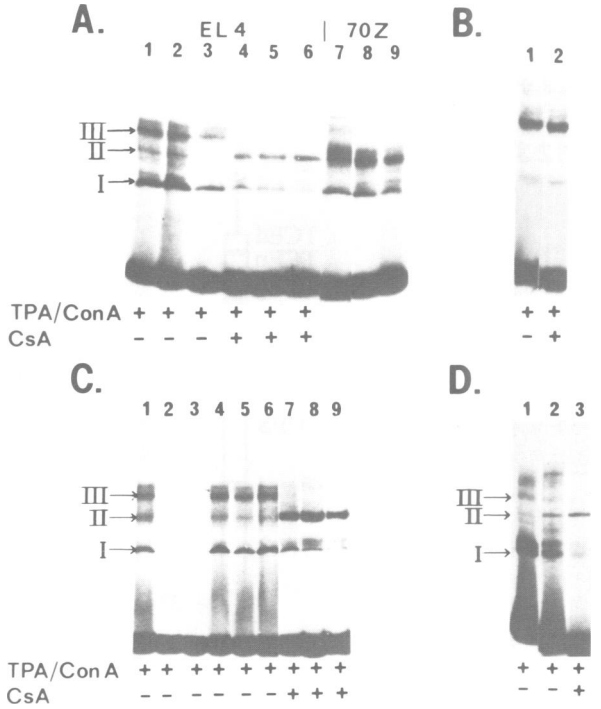


Fig. 3. Identification of CsA-sensitive Pu-box factors by electrophoretic mobility shift assays. **A.** Pu-box$_d$ binding proteins are missing in extracts from CsA-treated E14 cells. EMSA of Pu-box$_d$ probe (from −265 to −293, containing *Hin*dIII linkers) with extract from TPA/ConA-induced E14 cells (lanes 1−3), induced and CsA-treated E14 cells (lanes 4−6) or LPS-induced 70Z pre-B cells (lanes 7−9). In the assays, protein was used from the first lanes (1, 4 and 7), central (lanes 2, 5 and 8) or last fractions (lanes 3, 6 and 9) of the 0.4 M KCl peak from a heparin−agarose column. **B.** The binding of TCEd factors is not affected by CsA. EMSA of a TCEd DNA probe (−191 to −215) with protein from the 0.4 M KCl peak of a heparin−agarose column run with extracts from induced E14 cells (lane 1) or from induced and CsA-treated E14 cells (lane 2). **C.** The CsA-sensitive Pu-box factors correspond to specific, Pu-box DNA binding proteins. EMSA of Pu-box$_d$ DNA with protein from the 0.4 M peak of a heparin−agarose column run with protein extracts from induced cells (lanes 1−6) or induced and CsA-treated cells (lanes 7−9). For competition, the following unlabelled oligonucleotides were added: 5 ng (lanes 2 and 8) or 15 ng Pu-box$_d$ (lanes 3 and 9), 15 ng UPS (containing IL-2 enhancer DNA from −64 to −96; lane 4), 15 ng TCEd or 15 ng of a mutated TCEd (TCEd DNA with two T to G transversions at −202 and −203, and two G to T transversions at −205 and −207, respectively; lanes 5 and 6). **D.** Homologous factors bind to the distal and proximal Pu-boxes. EMSA of a proximal Pu-box$_p$ probe (from position −120 to −145; lanes 2 and 3) and of a distal Pu-box$_d$ probe (lane 1) with protein from the 0.4 M KCl peak of a heparin−agarose column run with extract from induced E14 cells (lanes 1 and 2) or from induced and CsA treated E14 cells (lane 3).

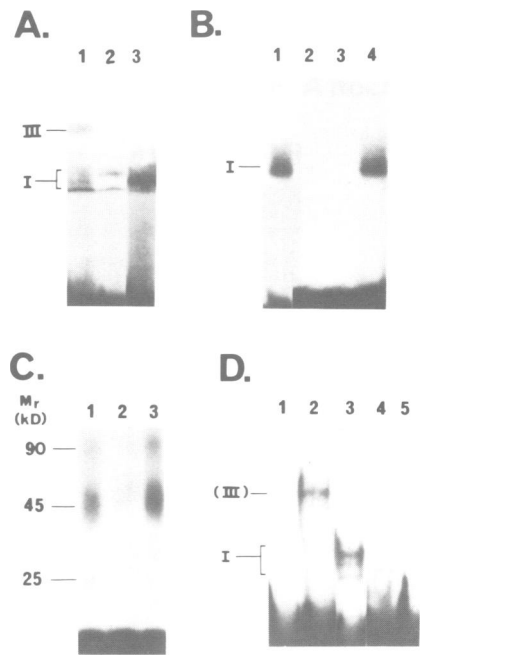


Fig. 4. Molecular characterization of isolated Pu-box factors. **A** and **B.** EMSA of Pu-box factors isolated by chromatography on a Pu-b$_d$ DNA affinity column from two different whole cellular protein extracts. Extract (A) contained all three factors, extract (B) factor I only. The protein of the 0.4 M KCl peak from heparin−agarose columns run with extracts from induced E14 cells was passed through affinity columns containing ligated Pu-b$_d$ oligonucleotides (−265 to −293). After extensive washing, the bound protein was eluted with 0.6 M KCl. Aliquots were incubated in EMSAs with a Pu-b$_d$ probe and the following competitor DNAs: **A.** Lane 1: no competitor, lane 2: 50 ng Pu-b$_d$, lane 3: 50 ng TCEd. **B.** Lane 1: no competitor, lane 2: 50 ng Pu-b$_d$, lane 3: 50 ng Pu-b$_p$, lane 4: 50 ng TCEd. **C.** Polypeptides of 45 kd are the main component of Pu-box factor I. Pu-box factors (from the preparation of Figure 4B) were incubated with a bromodeoxyuridine-substituted Pu-b$_d$ probe, UV irradiated for 15 min and fractionated on a SDS−10% polyacrylamide gel for 3 h at 250 V/10 cm. Lanes 2 and 3: incubation with a 100-fold excess of unlabelled Pu-b$_d$ or TCEd DNA, respectively. **D.** EMSA analysis of Pu-box factors after fractionation on a denaturing SDS−polyacrylamide gel and renaturation. The protein extract used in the EMSA experiment of Figure 3A (lane 2) was separated on a SDS−10% polyacrylamide gel. After the run, the gel was cut into five slices, containing the polypeptides >90 kd (slice 1), 50−90 kd (2), 30−50 kd (3), 20−30 kd (4) and <20 kd (5). The proteins were extracted, renatured and incubated in EMSAs with a Pu-b$_d$ probe. The complexes are indicated according to the position of complexes generated from non-denatured protein which was fractionated in the same EMSA gel (not shown).

as normal (Figure 3A and C). In a few incubations only complex I was formed (Figure 4B). The complexes I, II and III disappeared, or were found in reduced amount, after competition with a 25- to 50-fold molar excess of cold Pu-b$_d$ or Pu-b$_p$ DNA, but not with the same amount of TCEd or UPS DNA (compare lanes 2 and 3 with lanes 4−6 in Figure 3C). Incubation of Pu-b$_d$ DNA with extracts from CsA-treated El4 cells resulted in the formation of complex II only, which could not be competed by a 50-fold excess of Pu-box DNA (Figure 3A, lanes 4−6 and 3C, lanes 7−9). The complexes I and III were either absent or, in the case of complex I, sometimes present in a reduced amount. The formation of complexes I and II was also observed with extracts from 70Z pre-B cells, whereas complex III was only observed after incubation with El4 cell extract (Figure 3A).

Molecular properties of Pu-box factors

For the molecular analysis of Pu-box factors, we isolated them from extracts of induced El4 cells by affinity chromatography on Pu-b$_d$ DNA columns (see Materials and methods). All three Pu-box factor complexes were recovered (Figure 4A and our unpublished results). UV crosslinking of the most prominent factor I to a bromodeoxyuridine-substituted Pu-b$_d$ probe and subsequent electrophoresis through a SDS−polyacrylamide gel resulted in the detection of polypeptides of ~45 kd and, in trace amounts, polypeptides of ~25 and 90 kd (Figure 4C). Factor I contacts both Pu-boxes at identical positions. In methyla-

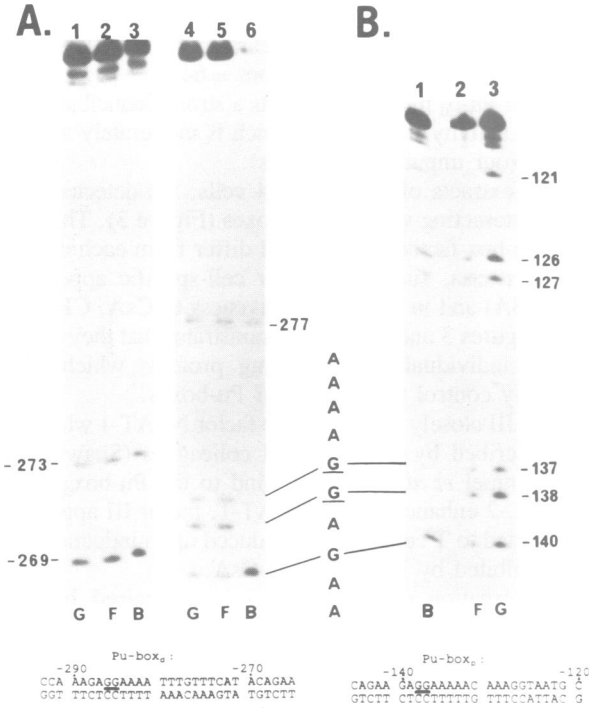


Fig. 5. Pu-box factor I contacts both Pu-boxes at identical nucleotides. Methylation interference analysis. ^{32}P-labelled probes of distal Pu-b$_d$ (**A**) and proximal Pu-b$_p$ (**B**) were partially methylated and incubated with isolated Pu-box factors I (Figure 4B) in EMSAs. The bound (B) and free DNAs (F) were blotted on DEAE filters, eluted, cleaved by piperidine and analysed on denaturing 12.5% polyacrylamide gels in parallel with a G-specific sequencing reaction (G). Fluorograms are shown for both strands of Pu-b$_d$ (**A**, lanes 1–3, noncoding DNA strand; lanes 4–6, coding strand) and of the coding strand of Pu-b$_p$ (B, lanes 1–3). The underlined G residues within the Pu-box sequences indicate the G nucleotides essential for protein binding.

tion interference experiments, the methylation of two central G residues within the Pu-box 'core' at positions −285 and −286 of Pu-b$_d$; −137 and −138 of Pu-b$_p$ interfered with the binding of complex I proteins, whereas the methylation of further Gs had no effect on factor binding (Figure 5A and B).

Fractionation of Pu-box factor proteins on denaturing SDS–polyacrylamide gels and subsequent EMSA analysis of isolated and renatured polypeptides resulted in the reconstitution of complexes which, by electrophoretic mobility, resembled the original Pu-box factors (Figure 4D). Mixing experiments with fractions extracted from different gel slices did not reconstitute new complexes (not shown). These results suggest that the Pu-box factors bind individually to their DNA target sequences and do not represent heterodimers that consist of polypeptides of very different sizes.

The role of Pu-boxes in the IL-2 enhancer activity

Removal of the distal Pu-box sequences (from −293 to −263) lowers the IL-2 enhancer activity to ~15% of the wild-type level, and replacement of the proximal Pu-box by *Hin*dIII linkers has a similarly negative effect (Table I, Serfling *et al.*, 1989). When both Pu-boxes are deleted, as in the construct pIlCAT2/1+ΔPu-b, the linked CAT gene is not inducible in El4 cells, and its weak constitutive expression is unaffected by CsA (Table I). This demonstrates that the two Pu-boxes are important functional sequence motifs which play an essential role in the establishment of IL-2 enhancer activity.

Table I. The role of Pu-boxes in the IL-2 enhancer activity

	Constructs	IL-2 sequences	Ratio of induction (% of acetylation)		No. of experiments
A.					
	pIlCAT 2/1+	−293/−7	90 (80)		3
	pIlCAT 2/1+A	−263/−7	13 (14)		3
B.					
	pIlCAT 2/1+	−293/−7	37 (43)		5
	pIlCAT 2/1+	−293/−156	6 (7)		5
	Δ−156/−104	−104/−14			
C.			−CsA	+CsA	
	pIlCAT 2/1+	−293/−7	12.5 (32)	1.5 (2)	3
	pIlCAT 2/1+	−250/−156			
	ΔPu-box	−104/−14	1 (0.4)	1 (0.5)	3

A. and B. The deletion of one Pu-box (A.: Pu-b$_d$; B.: Pu-b$_p$) leads to a drastic reduction of inducible IL-2 enhancer activity.
C. The deletion of both Pu-boxes abolishes the IL-2 enhancer activity. For the construction of pIlCAT plasmids see Serfling *et al.* (1989) and Materials and methods. The ratio of induction corresponds to the percentage of [^{14}C]chloramphenicol acetylation by the extracts from induced cells divided by those of uninduced cells. The % of acetylation corresponds to the acetylated chloramphenicol determined for the TPA/ConA-stimulated cells.

How does CsA inhibit the binding of Pu-box factors to the IL-2 enhancer?

To determine how CsA prevents the binding of Pu-box factors to the IL-2 enhancer, we pre-incubated cellular extracts with CsA. Since the generation of Pu-box factor complexes was not impaired the effect of CsA is not simply due to the binding of CsA to *trans*-acting factors. There are many other possible ways by which CsA might impair factor binding, e.g. (i) by blocking the synthesis of factors at the transcriptional or translational level, or (ii) by inhibiting co- and post-translational protein modification events. As shown in Figure 6A, inhibition of protein synthesis by cycloheximide (CHX) during the induction resulted in the loss of factor binding to the Pu-boxes. But unlike CsA, CHX also inhibited the binding of AP-1 like factors and changed factor binding to the TCE motifs. EMSA experiments demonstrated that CHX treatment (10 μg/ml) repressed the formation of Pu-box factor complex III and, in part, of complexes I and II (Figure 6B). A similar effect was detected for the transcription inhibitor actinomycin D (ActD) (Figure 6C). This suggests that the CsA-sensitive Pu-box factors are newly synthesized upon induction, and that CsA affects their synthesis. Alternatively, the synthesis of proteins involved in the modification (or activation) of Pu-box factors could be inhibited by CHX and ActD. However, so far we were unable to detect any co- or post-translational modifications of Pu-box factors (our unpublished results).

Discussion

Our data indicate that the two Pu-boxes of the murine IL-2 enhancer are CsA-responsive DNA elements (Figure 1). This is in agreement with studies of Emmel *et al.* (1989) in which the binding site for the factor NFAT-1 appeared to be a CsA-

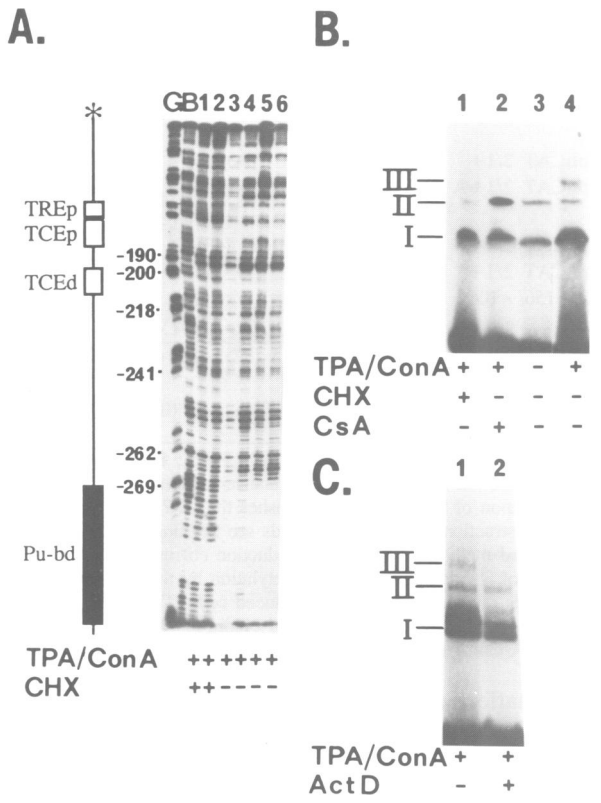


Fig. 6. The inhibition of protein and RNA synthesis impairs the DNA binding of Pu-box factors. **A**. Cycloheximide (CHX) treatment suppresses the binding of factors to the Pu-boxes. DNase I footprint protection. The *Hin*dIII−*Bam*HI fragment of pIICAT2/1+ (see Figure 1) labelled at the *Hin*dIII site was used as probe. Lanes 1 and 2: 25 μg (lane 1) and 60 μg protein (lane 2) from the central fraction of the 0.4 M KCl peak of a heparin−agarose column run with extract from E14 cells treated for 4 h with TPA/ConA and 10 μg/ml CHX. Lanes 3 and 4: 25 μg (3) and 60 μg protein (4) of a parallel culture of TPA/ConA-induced E14 cells. Lanes 5 and 6: 25 μg (5) and 60 μg protein (6) of a second, but non-parallel culture of induced E14 cells. **B** and **C**. CHX and ActD inhibit the generation of CsA-sensitive Pu-box factor complexes. **B**. CHX treatment. EMSA of Pu-b_d DNA with protein from the central fraction of a 0.4 M KCl column peak of extracts from induced E14 cells (lane 4), uninduced E14 cells (lane 3), and from induced and CsA-treated cells (lane 2) or induced and CHX-treated E14 cells (10 μg/ml CHX; treated for 4 h in the presence of TPA/ConA) (lane 1) **C**. ActD treatment. EMSA of Pu-b_d DNA with protein from the central fraction of a 0.4 M KCl column peak of extracts from induced E14 cells (lane 1) and of induced and ActD-treated cells (lane 2; 5 μg/ml ActD for 4h in the presence of TPA/ConA).

sensitive motif of the human IL-2 enhancer. The NFAT-1 binding site corresponds to the Pu-b_d of the murine IL-2 enhancer.

The Pu-boxes may not be the only CsA-responsive enhancer DNA elements of the IL-2 enhancer. Emmel *et al.* (1989) have reported that deletions of single protein binding motifs from the human IL-2 enhancer do not abolish the enhancer's sensitivity to CsA. But in all their deletion mutants one Pu-box was left, and this might be the reason for the remaining CsA sensitivity of enhancer mutants. In our latest transfection experiments, CsA did not impair the inducible activity of tetramers spanning the whole central enhancer fragment from positions −156 to −250 (Figure 1A) (our unpublished results). In addition to the TCEd, this large segment harbours numerous protein binding sites which are thought to be involved in the establishment of enhancer activity (Serfling *et al.*, 1989). However, a second CsA-responsive enhancer target appears to be the UPS motif which contains the sequences from −64 to −94. Like the TCEd and Pu-b_d motifs, it exhibits a strong inducible proto-enhancer activity in El4 cells which is moderately affected by CsA (our unpublished results).

In the extracts of induced E14 cells, we detected three factors interacting with the Pu-boxes (Figure 3). These so-called Pu-box factors I, II and III differ from each other in several criteria, such as in their cell-specific appearance (Figure 3A) and in their responsiveness to CsA, CHX and ActD (Figures 3 and 6). This demonstrates that they correspond to individual DNA binding proteins which could differently control the activity of Pu-boxes.

Factor III closely resembles the factor NFAT-1 which has been described by Crabtree and colleagues (Shaw *et al.*, 1988; Emmel *et al.*, 1989) to bind to the Pu-box$_d$ of the human IL-2 enhancer. Like NFAT-1, factor III appears to be restricted to T cells, rapidly induced upon induction, and to be inhibited by low doses of CsA.

The formation of factor I is also impaired by CsA (Figure 3A). As shown in Figure 4D, factor I seems to consist of several factors differing slightly in their electrophoretic mobilities. Since factor I also occurs in B cells (Figure 3A) one may assume that it corresponds to the factor described by Pettersson and Schaffner (1987) which binds to a purine rich sequence of the SV40 enhancer and contributes to the enhancer's activity in lymphoid cells. The 'core' sequence of the SV40 Pu-box, TGAAGAGGAA, matches the IL-2 Pu-box core in eight out of 11 nucleotides (Table II) and is also part of the lymphotropic papovavirus enhancer (Mosthaf *et al.*, 1985). Using nuclear extracts from BJA-B cells, the B cell factor was found to contact all nine purine nucleotides of the Pu-box core sequence and in particular the underlined G residues (Pettersson and Schaffner, 1987). The IL-2 Pu-box factor(s) I contacts the IL-2 Pu-box core in a similar, but not identical manner since they do not interact with the double underlined G residue of the Pu-box core (Figure 5). Moreover, in EMSA experiments IL-2 Pu-box DNA competes with SV40 DNA with low efficiency for complex formation (Pettersson and Schaffner, 1987). Thus, it seems likely that the activity of the SV40 Pu-box is controlled by multiple protein factors as well, and one of them seems to be related in its DNA binding properties to the Pu-box factor(s) I of the IL-2 enhancer. A cDNA coding for (one of) the Pu-box factor(s) of the SV40 enhancer has recently been isolated (Klemsz *et al.*, 1990). It codes for a protein of ~27 kd which is related to proteins of the *ets* oncogene family and restricted to B cells and macrophages.

In contrast to factors I and III, the binding of factor II to the Pu-boxes is not impaired by CsA. Instead, in extracts from CsA-treated El4 cells, factor II often forms a very prominent complex (see lanes 7−9 in Figure 3C) which can be enriched by affinity chromatography on Pu-b_d DNA columns (unpublished results). However, the formation of complex II by extracts from CsA-treated cells is not impaired by a 25- to 75-fold excess of Pu-box DNA (Figure 3C, lanes 7−9). Thus, it remains to be shown whether complex II corresponds to an unspecific DNA binding factor or, as we speculate, to a factor involved in the repression of Pu-boxes in uninduced and CsA-repressed cells.

Table II. The occurrence of Pu-box like sequences within the 5′ regions of several lymphokine genes, the LTR of HIV and the SV40 enhancer

Gene	Species	Sequence	Position
IL-2			
distal	man	A A G G A G G A A A A	−284/−274
proximal		A A G − A G G A A A A	−138/−129
distal	mouse	A A G − A G G A A A A	−290/−281
proximal		A A G − A G G A A A A	−142/−133
IL-3	mouse	A A G G A A G A A G A	−208/−198
IL-4	man mouse	C A G G A G A A A A A	−127/−137
IL-6	man	A A A G A A A A A A A	−318/−328
GM-CSF			
distal	man	C C T A G G G A A A A	−184/−174
proximal		A C A G A G G A A A T	−68/−78
proximal	mouse	A C A G A G G A A A T	−72/−82
HIV-1	man	A A G G A G G A A C A	−270/−260 (1.)
		A A G G A G A G A A C	−268/−258 (2.)
SV40	monkey	A A − G A G G A A C T	315/306

The transcription of all indicated lymphokine genes is inhibited by CsA. The sequence data are from Fujita *et al.* (1986: for the IL-2 and the 1. HIV sequence), Miyatake *et al.* (1985a: IL-3), Eder *et al.* (1988: human IL-4), Otsuka *et al.* (1987: murine IL-4), Miyatake *et al.* (1985b: GM-CSF), Peterlin *et al.* (1988: 2. HIV sequence) and Pettersson and Schaffner (1987: SV40).

To date we do not know by which mechanisms CsA treatment leads to the loss of Pu-box factor binding. Since CHX and ActD also impair the factor binding (Figure 6A−C) we assume that CsA suppresses the synthesis of factors. An alternative possibility is that cyclophilin, the major cellular target for CsA, is involved in the maturation and activation of Pu-box factors. Cyclophilin has recently been described to be a *cis−trans* peptidyl-prolyl isomerase, and its activity is efficiently inhibited by low doses of CsA (Fischer *et al.*, 1989; Takahashi *et al.*, 1989). FK 506, another potent inhibitor of lymphokine synthesis, binds to cytosolic enzymes of the same specificity (Harding *et al.*, 1989; Siekierka *et al.*, 1989) and also inhibits the DNA binding of Pu-box factors (our unpublished results). This suggests that *cis−trans* peptidyl-prolyl isomerases could be involved in the control of lymphokine gene activity and, possibly, in an activation step of Pu-box factors. However, in EMSA experiments we have not detected any influence of purified cyclophilin from porcine kidney (Fischer *et al.*, 1989) on the DNA binding of (preformed) Pu-box factors (our unpublished results). In Jurkat lymphoma cells, additional CsA binding proteins (of 25 kd, 45 kd and 70 kd) have been detected (Foxwell *et al.*, 1988; Ryffel *et al.*, 1989), and it will be interesting to see whether they exert cyclophilin-like activities.

Pu-box like sequence motifs are also part of the 5′ regulatory regions of other lymphokine genes and of the LTR sequences of HIV (Table II). In DNase I footprint protection experiments we have detected the binding of CsA-responsive factors to a Pu-box like motif within the upstream region of the human IL-4 gene (Table II; our unpublished results). Since CsA suppresses the expression of >10 genes upon mitogenic activation of human T cells (Zipfel *et al.*, 1989) we believe that the Pu-box factors play a crucial role not only in the expression of the IL-2 gene, but in the expression of many other lymphokine genes as well.

Materials and methods

Cells, DNA transfections and CAT assays

Murine El4 lymphoma cells were grown in RPMI medium supplemented with 5% fetal calf serum to a density of $\sim 4 \times 10^5$ cells/ml. 15 μg DNA of constructs (purified by two CsCl gradient centrifugations) was transfected into about 2.5×10^7 El4 cells in a final volume of 1.8 ml by the DEAE−dextran method according to Luthman and Magnusson (1983). After the transfection, the cells were incubated for 3 min in 15% dimethylsulphoxide and, 20 h later, they were divided. One third was induced by TPA/ConA (10 ng/2.5 μg per ml), one third by TPA/ConA and CsA (0.1 μg/ml; Sandoz, Basel), and one third was used as an uninduced control. The cells were incubated for 20 h in RPMI, sonicated and their CAT activities were measured as described (Gorman *et al.*, 1982; Serfling *et al.*, 1989).

DNA cloning

All recombinant DNA work was done according to standard recombinant DNA procedures (Maniatis *et al.*, 1982). The construction of many recombinant plasmids used in this work has been described (Serfling *et al.*, 1989), except for the following:

4×Pu-b$_d$. Chemically synthesized oligonucleotides containing the IL-2 enhancer sequences from position −264 to −292 and sticky ends for *Bam*HI and *Sal*I were polymerized by ligation in a small volume and fractionated on a low melting agarose gel. Multimers of four copies (and more) were extracted from the gel and introduced into pBLCAT2 (Luckow and Schütz, 1987). Three copies were cloned in the direction of transcription, one (the second from the proximal end) in the opposite direction.

5×TCEd+Il2 promoter. The tk promoter of 5×TCEd (see Serfling *et al.*, 1989) was removed by *Bam*HI−*Bgl*II digestion and religation. *Xba*I linkers were added to an IL-2 fragment spanning the promoter sequences from position +44 (at the *Pst*I site) to −104 (*Acc*I site), and it was introduced into the *Xba*I site of the promoterless 5×TCEd.

pIICAT2/1+ΔPu-b. The *XbaI*/*Dra*I fragment from pIICAT2/1+B (containing the IL-2 enhancer fragment from −250 to −168; Serfling *et al.*, 1989) and the *Dra*I−*Bgl*II fragment from pIICAT2/1+ Δ−156/−104 (containing the sequences from −168 to −156 and −104 to −14, and five *Hin*dIII linkers and one *Sal*I linker in between; Serfling *et al.*, 1989) were introduced into a tk promoterless pBLCAT2 plasmid.

Preparation of DNA binding proteins

Whole cell extracts were prepared as described (Angel *et al.*, 1987; Imagawa *et al.*, 1987) from packed deep-frozen E14 cells stimulated for 4 h by TPA/Con A (10 ng/2.5 μg per ml). 70Z pre-B cells were stimulated by 5 μg/ml lipopolysaccharide for 20 h. In the experiments with CsA or other inhibitors, parallel cultures were used for the extract preparation. The extracts were passed over a heparin−agarose column, and individual fractions of the 0.4 M KCl column peak were used in the DNase I and EMSA experiments. For the isolation of Pu-box factors, the protein from the 0.4 M KCl peak was passed over a DNA affinity column prepared according to Kadonaga and Tjian (1986) with ligated Pu-box$_d$ oligonucleotides.

DNA−protein binding experiments

For the DNase I footprint experiments, end-labelled DNA probes were prepared with AMV reverse transcriptase, and ~5000 c.p.m. (= ~0.1 ng) of 300 bp-long probes were incubated for 60 min on ice with 5−200 μg protein (from the 0.4 M KCl fraction of heparin−agarose column) in a total volume of 50 μl containing 1 μg polydI·dC. After 2 min incubation at room temperature, the samples were DNase I treated for 2 min, phenolized and precipitated (see Angel *et al.*, 1987; Serfling *et al.*, 1989). The radioactive samples were fractionated on 6% polyacrylamide, 42% urea sequencing gels.

In the electrophoretic mobility shift assays (EMSA), chemically synthesized and annealed double-stranded oligonucleotides were used. About 5000 c.p.m. (= 0.2 ng) of ^{32}P-labelled probes were incubated in a final volume of 15 μl with 4 μg protein from the 0.4 M KCl peak of the heparin−agarose column, along with 4 μg polydI·dC in 20 mM KCl and 4% Ficoll for 20 min on ice (Schreiber *et al.*, 1988). The samples were fractionated on native 4% polyacrylamide gels at 200 V/15 cm at room temperature.

For the size determination of factor I polypeptides (Figure 4C), factors isolated on a DNA affinity column (see above) were incubated with a bromodeoxyuridine-substituted Pu-b$_d$ probe (Böhnlein *et al.*, 1988), UV irradiated for 15 min and fractionated on a 10 cm long SDS−10%

polyacrylamide gel for 3 h at 250 V. For the same purpose (see Figure 4D), the protein from the 0.4 M KCl peak of a heparin–agarose column run with extract from induced E14 cells was fractionated on a SDS–10% polyacrylamide gel. The gel was cut into five slices. The protein of slices was extracted, renatured according to Briggs *et al.* (1986) and tested for the binding to Pu-box DNA in EMSA assays (Schreiber *et al.*, 1988).

In the methylation interference experiments (Sen and Baltimore, 1986), chemically synthesized single-stranded oligonucleotides were 5′-labelled with [γ^{32}P]ATP, annealed with the complementary, unlabelled oligonucleotide, and partially methylated at their G residues (Maxam and Gilbert, 1980). The methylated probe was incubated with Pu-box factors, followed by fractionation on EMSA gels. The DNA–protein complexes and the free DNA were blotted on DEAE-paper, extracted (Baeuerle and Baltimore, 1989), cleaved by 1 M piperidine and fractionated on 12% polyacrylamide, 42% urea sequencing gels (Maxam and Gilbert, 1980).

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References

Angel,P., Imagawa,M., Chiu,R., Stein,P., Imbra,R.J., Rahmsdorf,H.J., Jonat,C., Herrlich,P. and Karin,M. (1987) *Cell*, **49**, 729–739.

Baeuerle,P.A. and Baltimore,D. (1989) *Genes Dev.*, **3**, 1689–1698.

Böhnlein,E., Lowenthal,J.W., Siekevitz,M., Ballard,D.B., Franza,B.R. and Greene,W.C. (1988) *Cell*, **53**, 827–836.

Briggs,M.R., Kadonaga,J.T., Bell,S.P. and Tjian,R. (1986) *Science*, **234**, 47–52.

Brunvand,M.W., Schmidt,A. and Siebenlist,U. (1988) *J. Biol. Chem.*, **263**, 18904–18910.

Crabtree,G.R. (1989) *Science*, **243**, 355–361.

Drugge,R.J. and Handschuhmacher,R.E. (1988) *Transplantation Proc. XX*, Suppl. 2, 301–309.

Durand,D.B., Bush,M.R., Morgan,J.G., Weiss,A. and Crabtree,G.R. (1987) *J. Exp. Med.*, **165**, 397–407.

Durand,D.B., Shaw,J.P., Bush,M.R., Replogle,R.E., Belagaje,R. and Crabtree,G.R. (1988) *Mol. Cell. Biol*, **8**, 1715–1724.

Eder,A., Krafft-Czepa,H. and Krammer,P. (1988) *Nucleic Acids Res.*, **16**, 772.

Elliott,J.F., Lin,Y., Mizel,S.B., Bleackley,R.C., Harnish,D.G. and Paetkau,V. (1984) *Science*, **226**, 1439–1441.

Emmel,E.A., Verweij,C.L, Durand,D.B., Higgins,K.M., Lacy,E. and Crabtree,G.R. (1989) *Science*, **246**, 1617–1620.

Fischer,G., Wittmann-Liebold,B., Lang,K., Kiefhaber,T. and Schmid,F.X. (1989) *Nature*, **337**, 476–478.

Foxwell,B.M.J., Hiestand,P.C., Wenger,R. and Ryffel,B. (1988) *Transplantation*, **46**, 35S–40S.

Fujita,T., Shibuya,H., Ohashi,T., Yamanishi,K. and Taniguchi,T. (1986) *Cell*, **46**, 401–407.

Gorman,C.M., Moffat,L.F. and Howard,B.H. (1982) *Mol. Cell. Biol*, **2**, 1044–1051.

Harding,M.W., Galat,A., Uehling,D.E. and Schreiber,S.L. (1989) *Nature*, **341**, 758–760.

Hoyos,B., Ballard,D.W., Böhnlein,E., Siekevitz,M. and Greene,W. (1989) *Science*, **244**, 457–460.

Imagawa,M., Chiu,R. and Karin,M. (1987) *Cell*, **51**, 251–260.

Kadonaga,J.T. and Tjian,R. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 5889–5893.

Kahan,B.D. (1989) *New Engl. J. Med.*, **321**, 1725–1738.

Klemsz,M.J., McKercher,S.R., Celada,A., van Beveren,C. and Maki,R.A. (1990) *Cell*, **61**, 113–124.

Krönke,M., Leonard,W.J., Depper,J.M., Arya,S.K., Wong-Staal,F., Gallo,R.C., Waldmann,T.A. and Greene,W. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 5214–5218.

Luckow,B. and Schütz,G. (1987) *Nucleic Acids Res.*, **15**, 5490.

Luthman,H. and Magnusson,G. (1983) *Nucleic Acids Res.*, **11**, 1295–1308.

Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning. A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Maxam,A.M. and Gilbert,W. (1980) *Methods Enzymol.*, **65**, 499–560.

Mercurio,F. and Karin,M. (1989) *EMBO J.*, **8**, 1455–1460.

Miyatake,S., Yokota,T., Lee,F. and Arai,K. (1985a) *Proc. Natl. Acad. Sci. USA*, **82**, 316–320.

Miyatake,S., Otsuka,T., Yokota,T., Lee,F. and Arai,K. (1985b) *EMBO J.*, **4**, 2561–2568.

Mosthaf,L., Pawlita,M. and Gruss,P. (1985) *Nature*, **315**, 597–600.

Otsuka,T., Villaret,D., Yokota,T., Takebe,Y., Lee,F., Arai,N. and Arai, K.-I. (1987) *Nucleic Acids Res.*, **15**, 33–344.

Pettersson,M. and Schaffner,W. (1987) *Genes Dev.*, **1**, 962–972.

Peterlin,B.M., Calman,A.F., Kao,S.Y., Selby,M., Tong-Starksen,S. and Luciw,P.A. (1988) In *The Control of Human Retrovirus Gene Expression.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 45–57.

Renkawitz,R. (Ed.) (1989) *Tissue Specific Gene Expression.* VCH Verlagsgesellschaft Weinheim.

Ryffel,B., Foxwell,B.M.J., Mackie,A., Rodriguez,C. and Woerly,G. (1989) *7th International Congress of Immunology*, Berlin. Gustav Fischer Verlag, Stuttgart and New York, Abstr. 36–48.

Schreiber,E., Matthias,P., Müller,M.M. and Schaffner,W. (1988) *EMBO J.*, **7**, 4221–4229.

Sen,R. and Baltimore,D. (1986) *Cell*, **46**, 705–716.

Serfling,E., Barthelmäs,R., Pfeuffer,I., Schenk,B., Zarius,S., Swoboda,R., Mercurio,F. and Karin,M. (1989) *EMBO J.*, **8**, 465–473.

Shaw,J.P., Utz,P.J., Durand,D.B., Toole,J.J., Emmel,E.A. and Crabtree,G.R. (1988) *Science*, **241**, 202–205.

Shibuya,H. and Taniguchi,T. (1989) *Nucleic Acids Res.*, **17**, 9173–9184.

Shibuya,H., Yoneyama,M. and Taniguchi,T. (1988) *Int. Immunol.*, **1**, 43–49.

Siekierka,J.J., Hung,S.H.Y., Poe,M., Lin,C.S. and Sigal,N.H. (1989) *Nature*, **341**, 755–757.

Smith,K.A. (1988) *Science*, **240**, 1169–1176.

Takahashi,N., Hayano,T. and Suzuki,M. (1989) *Nature*, **337**, 473–475.

Toribio,M.L., Gutierrez-Ramoz,J.C., Pezzi,L., Marcos,M.A.R. and Martinez,A.C. (1989) *Nature*, **342**, 82–85.

Zipfel,P.F., Irving,S.G., Kelly,K. and Siebenlist,U. (1989) *Mol. Cell. Biol.*, **9**, 1041–1048.

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